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A placebo controlled observer blind immunocytochemical and histologic study of epithelium adjacent to anogenital warts in patients treated with systemic interferon alpha in combination with cryotherapy or cryotherapy alone

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Abstract

***Objective*—To examine biopsy specimens of tissue immediately adjacent to anogenital (AG) warts which had been treated with either cryotherapy plus subcutaneous interferon (IFN) alpha 2a or cryotherapy alone, for histological features of (*a*) human papilloma virus (HPV) infection (*b*) localised cellular immune responses, to further characterise any cellular immune infiltrates with tissue immunocytochemistry, and to relate any histological, immununocytochemical findings to the treatment response of nearby AG warts.**

***Design*—A randomised placebo controlled observer blind study.**

***Setting*—Genitourinary Medicine clinic, Department of Immunopathology, Royal Victoria Hospital, Belfast, N. Ireland.**

***Subjects*—Thirty patients with AG warts; 16 treated with IFN alpha 2a plus cryotherapy, and 14 treated with cryotherapy alone.**

***Outcome measures*—(1) Light microscopic features associated with HPV infection and local cellular immune responses. (2) Indirect immunofluorescence detection of the following cell surface markers: HLA DR, alpha one antitrypsin, CD1, CD3, CD4, CD8, CD22. (3) Clinical response of AG warts to treatment.**

***Results*—In pre-treatment biopsies only non specific indicators of HPV infection (acanthosis, 29/30 biopsies, and hyperkeratosis, 7/30 biopsies) were seen on light microscopy. Mononuclear cells were seen both throughout the upper dermis and centred around dermal blood vessels in 19/30 (63·3%) biopsies, and infiltrating into the epidermis in 12/30 (40%) biopsies. On indirect immunofluorescence CD3, CD8, CD4 antigen was detected on the surface of cells throughout the upper dermis in 24/29 (82·7%), 15/29 (51·7%), and 3/29 (10·3%), of biopsy specimens respectively. CD3 antigen, CD8 antigen and CD4 antigen was detected on the surface of cells infiltrating into the epidermis in 18/29 (62%), 7/29 (24·1%), and 6/29 (20·7%) of biopsy specimens respectively. CD1 antigen was seen on the surface of dendritic cells throughout the epidermis in all specimens; CD1 positive cells infiltrated into the upper dermis in 5/29 (17·2%). HLA DR was detected on the surface of dendritic cells throughout the epidermis in 22/29 (75·9%) of specimens, and on the surface of cells scattered both diffusely throughout the upper dermis and centred around dermal blood vessels in all specimens. Alpha one antitrypsin (A1AT) antigen was seen on the surface of cells in the upper dermis in 6/29 (20·7%) of biopsy specimens; no cells expressing CD22 surface antigen were seen. The nature of this local cellular immune response was not altered by treatment of nearby warts with either cryotherapy alone or cryotherapy plus systemic IFN alpha 2a, or related to the therapeutic outcome of these warts.**

***Conclusions*—(1) No convincing histological evidence of HPV infection was seen in epithelium surrounding AG warts. (2) A predominantly T cell-mediated immune response (the target of which is uncertain) was seen in this perilesional epithelium. (3) In the dosage regimens used in this study, treatment of AG warts with either systemic IFN alpha 2a plus cryotherapy or cryotherapy alone did not appear to augment localised cellular immune responses (against any presumed subclinical HPV infection) in epithelium surrounding AG warts.**

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Introduction

Local predominantly T lymphocytic infiltrates seen in wart biopsies, either stable, spontaneously regressing, or regressing after treatment, suggest that a host specific cell mediated immune response (CMIR) is important in eradication of these lesions.[1–11]

In patients with AG warts primary treatment failure or clinical recurrence after apparently successful treatment is often attributed to persistence of subclinical/latent HPV infection in clinically normal surrounding epithelium.[12] However, such studies have

detected HPV DNA by extraction DNA techniques such as Southern blotting; there is currently no direct evidence (histology, in situ hybridisation) to topographically localise HPV in tissue surrounding AG warts.

If HPV infection of epithelium adjacent to AG warts does occur, the ability or inability of the host to mount an effective local CMIR against virus infected cells in this tissue, may play an important role in determining AG wart-treatment outcome.

In patients with AG warts, IFN administered either locally or systemically is postulated to exert its therapeutic effects in part via stimulation of a CMIR against HPV-infected keratinocytes both within clinical warts and in epithelium surrounding these lesions.

The aims of this study were: (1) to determine if histologic evidence of HPV infection is seen in epithelium adjacent to AG warts, (2) to determine the nature of any immune infiltrates in this perilesional epithelium, (3) to determine if treatment of AG warts with cryotherapy or systemic IFN alpha influences nature of any immune infiltrates in the epithelium adjacent to these warts, (4) to determine if any local immune response detected in tissue surrounding AG warts is related to treatment outcome of these warts.

Materials and Methods

This study was approved by the Medical Ethics committee of Queens University, Belfast. Patients over the age of 16 years were recruited from genitourinary clinic attenders with newly diagnosed AG warts, and allocated by computerised randomisation charts to IFN/cryotherapy or placebo/cryotherapy treatment groups. Exclusion criteria were previous treatment for AG warts within the preceding three years, immunosuppression past or present for any reason including concurrent drug therapy, and pregnancy.

Before trial entry patients had urethral, cervical, vaginal, and rectal swabs taken, as appropriate, to exclude non specific urethritis and infection with gonorrhoea, *Chlamydia trachomatis*, candida, *Trichomonas vaginalis*, *Gardnerella vaginalis*, and venous blood for syphilis serology. Any infections detected were appropriately treated before trial entry.

According to treatment group allocation patients were given subcutaneous IFN alpha 2a, in doses of three million units, or placebo injections (normal saline), on 3 separate days in the first week. Thereafter patients had IFN, three million units, or placebo injections, twice weekly, and cryotherapy of AG warts once a week, for the following 6 weeks; a total of 7 weeks treatment in all. Cervical exophytic warts/subclinical preneoplasias were not specifically treated.

The study was observer blind; injections of IFN or placebo were administered by HL; cryotherapy, clinical assessment were performed independently by JH. At initial assessment and 8 week review patients had a clinical AG tract examination including proctoscopy, and excision biopsy of epithelium adjacent to a clinical AG wart. At 3 month review patients had clinical examination including proctoscopy.

Cryotherapy was performed with a standard cryoprobe, after prior application of KY jelly to the wart(s), for a single 60 second freeze. When required local anaesthetic (EMLA) cream was applied instead of KY gel. Patients were instructed to take saline baths twice daily until healing of the treated area had occurred.

Initial excision biopsy of perilesional epithelium was taken after local subcutaneous injection of 1% lignocaine solution from an area within 1 cm of a representative AG wart, and the site marked. At treatment completion (8 weeks), a further biopsy was performed adjacent to the site of the previous biopsy.

At 8 week review patients clinically clear of warts were asked to reattend 6 weeks later; those who had persistent warts were withdrawn from the clinical study. Patients were advised either to avoid sexual intercourse completely or if this was not possible, to use condoms during the study period.

Biopsy processing for histology and indirect immunofluorescence

Fresh tissue was processed as described by Michel,[13] mounted into OCT (Ames Division, Miles Laboratories Inc., Elkhart, Indiana) blocks; 5 μm sections were cut in a Brights cryostat (Brights Instrument Company, Huntingdon, England), at $-20°C$, and taken onto glass multispot slides. These slides were incubated for 12 hours at 37°C, and stored prior to staining in airtight plastic bags at $-20°C$.

Prior to indirect immunofluorescence staining sections were washed for 10 minutes in 0·01M PBS (8·5 g NaCl, 1·07 g Na_2HPO_4 – anhydrous, 0·39 g $NaH_2PO_4 \cdot 2H_2O$, in one litre distilled water, pH = 7·1) at room temperature. They were then incubated with appropriate primary antisera (table 1) in a moist incubation chamber at 37°C for 40 minutes. Slides were washed and rinsed for 10 minutes in 0·01M PBS and incubated with appropriate secondary fluorescein labelled conjugates (table 1) in a damp chamber at 37°C for 20 minutes.

After a further 10 minute wash in 0·01M PBS, sections were mounted in buffered glycerol (10 ml 0·01M PBS, 90 ml glycerol, 2·5 g 1,4 diazo bicyclo 222 octane—stored at 4°C in the dark) under glass cover slips for fluorescent microscopy. Appropriate controls were run for each tissue biopsy.

A Leitz Orthoplan (E Leitz Instruments Ltd., Luton) fluorescent microscope was used for section analysis. Quantitative grading of cell surface antigen expression was established viewing a representative high power field (HPF) within epidermis and dermis. The following grading system was used:

0	= No fluorescence staining	per HPF
+	= 1/2 cells showing fluorescent staining	
+ +	= 3–10 cells showing fluorescent staining	
+ + +	= > 10 cells showing fluorescent staining	

Table 1 Details of primary and secondary antisera used in indirect immunofluorescence analysis of perilesional biopsies

Manufacturer/code	*Cell specificity (peripheral blood/epithelium)*	*Animal*	*Monoclonal polyclonal*	*Dilution in PBS 0·01 M*
Primary antisera (anti human)				
Becton-Dickinson, UK Ltd., Cowley, Oxford				
1. HLA DR 7360	LC, BL, act TL, monocyte, macrophage	Mouse	Monoclonal	1/50
Dakopatts, DAKO Ltd., High Wycombe				
1. Pan B m708 (CD22)	BL	Mouse	Monoclonal	1/50
2. T3 m756 (CD3)	Early TL	Mouse	Monoclonal	1/20
3. T4 m716 (CD4)	HTL/ITL	Mouse	Monoclonal	1/10
4. T6 m721 (CD1)	LC, macrophage reticulum cells	Mouse	Monoclonal	1/50
5. T8 m707 (CD8)	CTL/STL	Mouse	Monoclonal	1/20
6. A1AT ao12	Non specific macrophage	Rabbit	Polyclonal	1/500
Secondary FITC conjugated antisera				
Dakopatts, DAKO Ltd., High Wycombe				
1. Swine anti rabbit Ig (F205)		Swine	Monoclonal	1/20
2. Rabbit anti mouse Ig (F232)		Rabbit	Monoclonal	1/20

LC = Langerhans cell; TL = T lymphocyte; BL = B lymphocyte; act TL = activated T lymphocyte; HTL/ITL = helper/inducer T lymphocyte; CTL/STL = cytotoxic/suppressor T lymphocyte; A1AT = alpha one antitrypsin.

Immunofluorescence reporting was done independently by a consultant histopathologist (MW). Antigen staining is reported in the results section as positive if three or more cells per HPF (ie + + or more) showed surface fluorescent staining.

Working dilutions of primary and secondary fluorescein conjugated antisera were determined in a procedure modified from that described by Thompson.[14]

Additional 5 μm frozen sections of each biopsy were stained with haematoxylin and eosin, and mounted with Ralmount (BDH Ltd., Poole, England) under glass cover slips for light microscopy. The presence of histological features including inflammatory cell dermal/epidermal infiltrates were noted and quantitatively graded as follows:

0 = absent
\+ = sparse
+ + = mild
+ + + = moderate
+ + + + = severe.

Histology reporting was independently by consultant pathologist (MW). Features are shown as positive in the results section if + + or more.

Table 2 Demographic data of patients in each treatment group

	IFN/CRYO	*PLAC/CRYO*
No of patients	16	14
Age (median/range)	25/18–59	26/18–47
Sex: number of males	8	4
number of females	8	10
AG warts:		
1. Number of patients with history of warts	0	1
2. Median wart duration prior to first attend (months)	2	2
3. Median number of warts	10	13
4. Median area/extent (cm^2)	1	1
5. % of patients with warts at a specific site:		
Males: a. preputial cavity/glans/c. sulcus	37·5	50
b. foreskin/shaft	25	25
c. perianal/anal	12·5	50
d. intraurethral	12·5	25
Females: a. introitus	25	33·3
a. vulva	67·5	80
c. vaginal	0	10
d. perianal/anal	50	70
Male sexual behaviour:		
1. Heterosexual	7	4
2. Homosexual	1	0
Female sexual behaviour:		
1. Heterosexual	8	10
a. vaginal i/c alone	7	8
b. vaginal/anal i/c	0	2
c virgin	1	0
Other AG tract infections:		
1. Cervix low grade preneoplasia	4	8
2. Males:		
a. nsu	3	2
b. *G vaginalis*	1	0
Total	4	2
3. Females:		
a. nsgi	2	2
b. *G vaginalis*	3	2
c. *T vaginalis*	1	0
d. candida	1	3
Total	5	5

i/c = intercourse; nsu = non specific urethritis; nsgi = non specific genital infection.

Results

Statistical analysis was by Mann Whitney U and chi square tests. Demographic data of patients in each treatment group are shown in table 2. There were no significant differences between groups.

Histological findings are summarised in table 3. Mononuclear cells (lymphocytes, histiocytes, plasma cells) were mainly seen around superficial dermal blood vessels, less commonly scattered diffusely throughout the upper dermis, and extending into the lower epidermis.

Immunocytochemistry results are shown in table 4. No cells expressing CD22 markers were seen in any biopsy specimens. CD3 surface antigen expressing cells were present throughout the upper dermis, extending into the lower half of the epidermis, CD8 and CD4 surface antigen expressing cells were seen throughout the upper dermis and epidermis (fig 1). CD1 and HLA DR expression was seen on the surface of dendritic cells diffusely scattered throughout the epidermis (fig 2); CD1 expression was also seen on dendritic cells in the sub-basement membrane region, and HLADR expression on cells centred around superficial blood vessels and scattered diffusely throughout the upper dermis (fig 3). A1AT antigen was detected on the surface of occasional cells in the upper dermis.

There were no significant differences between pre and post treatment biopsy specimens, or in post treatment specimens between patients in either treatment group, on histology or immunofluorescence.

Table 3 Histological features of perilesional epithelium biopsy specimens taken from patients with primary AG warts treated with IFN plus cryotherapy or cryotherapy alone

	*Pre-Tx. Bx.**		*Post Tx. Bx.†*					
			IFN/Cryo		*Cryo*		*Total*	
	No	*%*	*No*	*%*	*No*	*%*	*No*	*%*
Acanthosis	29	96·7	13	81·3	11	78·5	53	88·3
Epidermal mono cell infiltrate	12	40	3	18·8	4	28·6	19	31·7
Hyperkeratosis	7	23·3	5	31·25	5	35·7	17	28·3
Epidermal spongiosis	5	16·6	1	6·25	0	0	6	10
Koilocytosis	1	3·3	0	0	0	0	1	1·7
Elongation rete pegs	1	3·3	0	0	0	0	1	1·7
Dermal mono cell infiltrate:								
1. Diffuse	4	13·3	2	12·5	1	7·1	7	11·7
2. Perivascular	15	50	8	50	9	64·2	32	53·3
3. Overall	19	63·3	10	62·5	14	71·3	39	65
Total no Bx	30	100	16	100	14	100	60	100

*Pretreatment biopsy.
†Posttreatment biopsy.

Table 4 Immunocytochemical analysis of cell surface antigen expression in perilesional epithelium biopsies from patients with primary AG warts treated with IFN plus cryotherapy or cryotherapy alone

	Pre treatment		*Post treatment*					
			IFN/Cryo		*Cryo*		*Total*	
	No	*%*	*No*	*%*	*No*	*%*	*No*	*%*
EPIDERMIS								
CD3	18	62	7	43·7	6	46·1	31	53·4
CD4	6	20·7	3	18·8	4	30·8	13	22·4
CD1	29	100	16	100	13	100	58	100
CD8	7	24·1	8	50	3	23·1	18	31
HLA DR	22	75·9	14	87·5	12	92·3	48	82·8
A1 AT	0	0	0	0	0	0	0	0
UPPER DERMIS								
CD3	24	82·7	15	93·75	12	92·3	51	87·9
CD4	3	10·3	2	12·5	3	23·1	8	13·8
CD1	5	17·2	5	31·3	2	15·4	12	20·7
CD8	15	51·7	12	75	7	53·8	34	58·6
HLA DR	29	100	15	93·8	13	100	57	98·3
A1 AT	6	20·7	3	18·8	0	0	9	15·5
Total no. Bx.	29	100	16	100	13	100	58	100

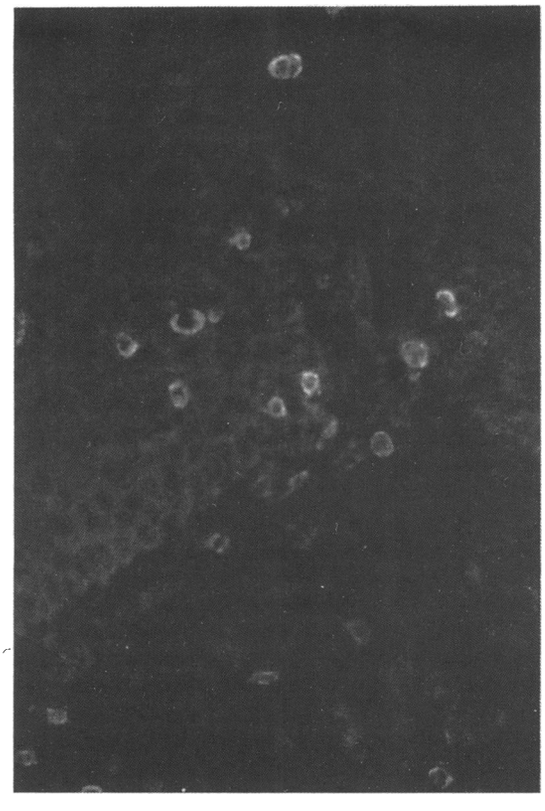

Figure 1 CD4 surface antigen expression on mononuclear cells in the papillary dermis and epidermis of epithelium adjacent to anogenital warts.

Table 5 compares histology/immunocytochemistry of post treatment biopsy specimens, between patients clear of warts, and those who had persistent warts, at treatment completion (8 weeks). Table 6 shows histology/immunocytochemistry of post treatment specimens from patients whose warts were clear at treatment completion, comparing those patients who had recurrence of warts, with those who remained clinically wart free, at 3 month review. There were no significant differences in post treatment biopsies between any of these groups.

This was part of a larger study comparing the therapeutic responses to treatment of primary AG warts with subcutaneous IFN alpha 2a plus cryotherapy, or cryotherapy alone, the results of which have been previously reported.[15]

Discussion

These results provide only non specific histological indicators of subclinical HPV infection in epithelium surrounding AG warts (that is, acanthosis and hyperkeratosis of the epidermis). Proof of HPV infection would require detection of specific histological indicators of HPV infection (that is, koilocytes,

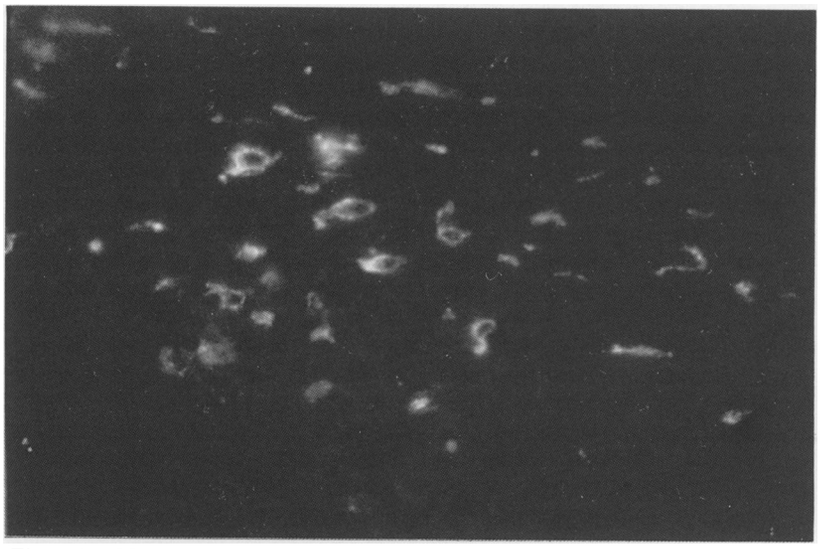

Figure 2 CD1 surface antigen expression on dendritic cells diffusely scattered throughout the epidermis of epithelium adjacent to AG warts.

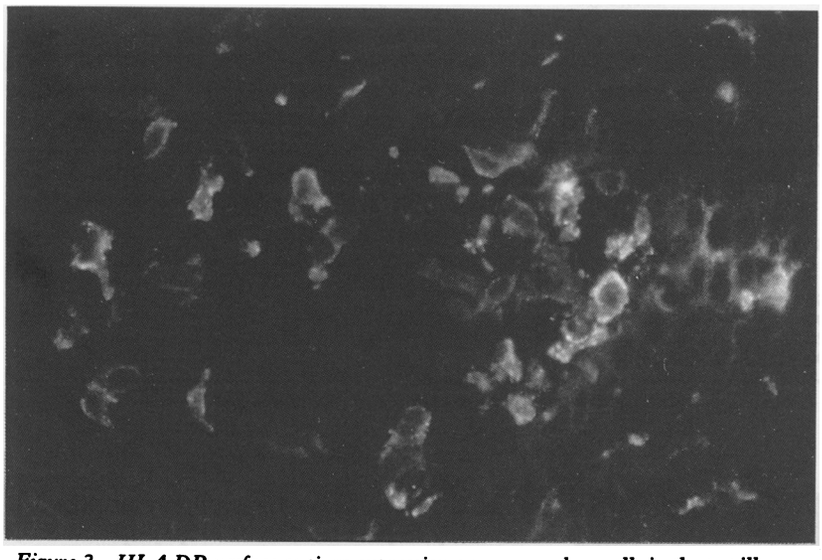

Figure 3 HLA DR surface antigen expression on mononuclear cells in the papillary dermis of epithelium adjacent to AG warts.

dyskeratosis), and direct topographic localisation of HPV DNA/RNA by a molecular hybridisation technique such as in situ hybridisation (ISH); simultaneous light microscopic/ISH analysis of normal AG tract epithelium in patients without AG warts would be necessary to establish the relevance of any findings.

The presence of a local specific CMIR (that is, HLA DR surface antigen expressing pan T, helper/inducer, cytotoxic/suppressor T lymphocytes, histiocytes, Langerhans cells) in epithelium adjacent to AG warts might be construed as evidence of host immune response against subclinical HPV infection in this area; whether this immune response is directed against HPV infection in this epithelium or an overspill of a local CMIR against nearby AG warts, is uncertain. Histology/immunocytochemistry of AG epithelium in controls, that is patients with no AG warts, and patients with subclinical HPV infection alone without concurrent clinical warts, might further clarify this issue.

Neither cryotherapy alone, nor systemic IFN alpha 2a treatment plus cryotherapy of AG warts, appeared to alter histologic/immunocytochemical findings in tissue surrounding these warts. Although further IFN dose response studies are required for confirmation, these findings do not support the theory that IFN therapy favourably influences outcome of AG warts by stimulating a local CMIR against subclinical/latent HPV infection in epithelium surrounding AG warts.

The nature of the local immune responses in epithelium adjacent to AG warts was not apparently related to clinical outcome of these warts. This suggests either that local immune response against subclinical/latent HPV infection in epithelium surrounding AG warts is not important in determining clinical outcome of these warts, or that local cellular immune infiltrates in perilesional tissue are only an insignificant overspill of the host CMIR against nearby AG warts.

Table 5 Comparison between post treatment perilesional biopsy specimens in relation to clinical outcome of AG warts at eight weeks

	Biopsies				
	Cure 8/52		*Persist 8/52*		
	No	*%*	*No*	*%*	*Sig/chi*
EPIDERMIS					
Mononuclear cell infiltrate (histology)	5	27·8	1	9·1	>0·1
Cell surface antigen expression					
CD3	7	38·9	6	54·5	>0·5
CD4	5	27·8	2	18·2	>0·5
CD1	17	94·4	11	100	>0·5
CD8	7	38·9	4	36·4	>0·5
HLA DR	16	88·7	10	90·9	>0·5
DERMIS					
Mononuclear cell infiltrate (histology)	10	55·6	8	72·7	>0·5
Cell surface antigen expression					
CD3	18	100	9	81·8	>0·5
CD4	5	27·8	0	0	>0·1
CD1	4	22·2	3	27·2	>0·5
CD8	9	50	6	54·5	>0·5
HLA DR	18	100	9	81·8	>0·5
A1 AT	2	11·1	0	0	>0·5
Total no Bx	18	100	11	100	

Table 6 Immunocytochemistry/histology of post treatment biopsies from patients clinically wart free at treatment completion (eight weeks): relation to wart outcome at three months

	Biopsies				
	Clear warts 8/52/recur at 3/12		*Clear 8/52/no recurrence*		
	No	*%*	*No*	*%*	*Sig/fisher*
EPIDERMIS					
Mononuclear infiltrate	2	22·2	2	33·3	0·42
Cell surface antigen expression					
CD3	6	66·7	1	16·6	0·07
CD4	4	44·4	1	16·6	0·25
CD1	9	100	5	83·3	0·4
CD8	4	44·4	2	33·3	0·38
HLA DR	9	100	5	83·3	0·4
DERMIS					
Mononuclear infiltrate	5	55·5	3	50	0·39
Cell surface antigen expression					
CD3	9	100	4	66·7	0·14
CD4	4	44·4	1	16·6	0·25
CD1	0	0	1	16·6	0·4
CD8	5	55·5	3	50	0·3
HLA DR	8	88·8	5	83·3	>0·5
A1 AT	1	11·1	1	16·6	>0·5
Total no Bx	9	100	6	100	

Additional factors such as small patient numbers, variation in distance of biopsy from clinical warts, may have influenced results in this study.

In summary this study provides only inconclusive circumstantial evidence of subclinical HPV infection, and direct evidence of a local CMIR, the target of which is uncertain, in epithelium surrounding AG warts. Subcutaneous IFN alpha treatment and/or cryotherapy of AG warts did not influence the histological features or nature of immune infiltrates in surrounding epithelium; these immune infiltrates did not appear to be related to clinical outcome of AG warts.

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